Transglycosylations catalysed by Y151M mutant of human salivary α-amylase (HSA)

Lili KANDRA[1]*, Gyöngyi GYÉMÁNT[1], Judit REMENYIK[2], Chandran RAGUNATH[3] & Narayanan RAMASUBBU[3]

[1] *University of Debrecen, Faculty of Sciences, Department of Biochemistry, P.O. Box 55, H-4010 Debrecen, Hungary; phone: ++ 36 52 512900, fax: ++ 36 52 512913, e-mail: kandra@tigris.unideb.hu*
[2] *Research Group for Carbohydrates of the Hungarian Academy of Sciences, P.O. Box 55, H-4010 Debrecen, Hungary*
[3] *Department of Oral Biology, University of Medicine and Dentistry of New Jersey, Newark, NJ 07103 USA*

Abstract: Biochemical and enzymatic characterisation has been achieved for the Tyr151Met (Y151M) mutant of human salivary α-amylase (HSA). Substantial transglycosylation capacity was detected in Y151M in addition to its hydrolytic activity. Y151M was capable of transferring maltose and maltotriose residues from a maltotetraose donor onto different 4-nitrophenyl glycosides resulting in the formation of 1-thio-β-D-glucosides, β- and α-D-glucosides and β-D-xylosides with DP 2-4 in yields up to 50%. Reactions were monitored using TLC, HPLC and MALDI-TOF MS. ^{1}H and ^{13}C NMR studies revealed that the Y151M preserved its stereo- and regio-selectivity. Glycosylation took place at position 4 of the glycosyl acceptors, resulting in the new α-1,4-glycosidic bonds exclusively.

Key words: human salivary α-amylase, Tyr151Met mutant, subsite map, transglycosylation, 4-nitrophenyl oligosides.

Abbreviations: HSA, human salivary α-amylase; PNP, *p*-nitrophenyl; α-Glc, α-D-glucopyranoside; β-Glc, β-D-glucopyranoside; 1-S-β-Glc, 1-thio-β-D-glucopyranoside; α-Man, α-D-mannopyranoside; α-Xyl, α-D-xylopyranoside; CNP, 2-chloro-4-nitrophenyl; BCF, bond-cleavage frequency.

Introduction

Enzyme-catalysed synthesis of oligosaccharides is a very attractive method because it allows the formation of well-defined oligosaccharides selectively without using any protection of hydroxyl groups. Many different oligosaccharides have already been synthesized by enzymatic transfer reactions. In addition, on the basis of the advances in genetic engineering, it is becoming possible to produce a wider range of enzymes on a large scale, extending the number of enzymes available for synthetic reactions. Despite the increasing work carried out with glycosidases, little is known about the structural requirements for the binding of sugar acceptors to the enzyme; this knowledge is essential to improve the synthetic utility of the methodology.

Retaining glycosidases are common biocatalysts used in chemo-enzymatic synthesis of oligosaccharides. Although the members of the α-amylase family use the α-retaining mechanism in their conserved catalytic site, it is striking to find that they vary widely in their substrate and product specificity. These differences can be attributed to differences in the architecture of their active site. Considerable effort has been made to study the application of the transglycosylation ability of retaining glycosidases as an alternative approach for the synthesis of chromogenic oligosaccharide substrates (USUI et al., 1993; ZENG et al., 2000; ENEYSKAYA et al., 2003). The great majority of the syntheses published in the literature are catalysed by exo-acting glycosidases. The ability of endo-acting enzymes to synthesize novel oligosaccharides has rarely been tested (USUI et al., 1993; ENEYSKAYA et al., 2003). In this paper we report on the synthesis of oligosaccharide glycosides catalysed by an endoenzyme, the mutant Y151M of the human salivary α-amylase (HSA). One possible shortcoming of this approach, however, is that the products of transglycosylation may be hydrolysed themselves by the enzyme during the course of reaction. The introduction of glycosynthases, mutant glycosidases, which lack the catalytic nucleophile and are therefore incapable of carrying out substrate hydrolysis in the presence of glycosyl fluoride donors, has overcome this problem for the synthesis of a number of β- and α-aryl oligosaccharides (JAKEMAN & WITHERS, 2002; OKUYAMA et al., 2002; WILLIAMS & WITHERS, 2002).

* Corresponding author

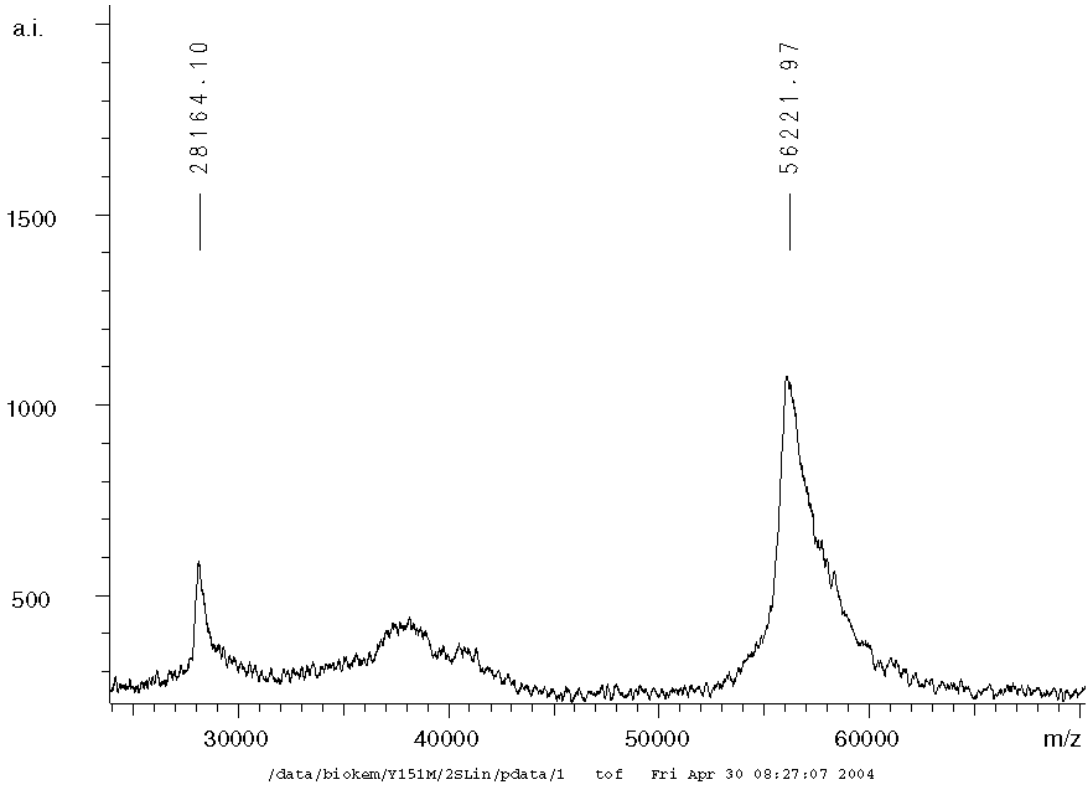


Fig. 1. MALDI-TOF spectrum of Y151M enzyme. First peak: $[M+2H]^{2+}$; second peak: $[M+H]^{+}$.

In this work a wide range of glycosyl acceptors, such as *p*-nitrophenyl α-D-glucopyranoside (PNP α-Glc), β-D-glucopyranoside (PNP β-Glc), 1-thio-β-D-glucopyranoside (PNP 1-S-β-Glc), α-D-mannopyranoside (PNP α-Man), and α-D-xylopyranoside (PNP α-Xyl), were utilized in transglycosylation experiments with Y151M mutant of HSA.

Material and methods

Substrates

The homologous maltooligomer substrate series (DP 4-8) was synthesised from cyclodextrins (FARKAS et al., 1997). The longer-chain-length of 2-chloro-4-nitrophenyl (CNP) maltooligosides in the range of DP 8-10 were prepared by a chemoenzymatic procedure using rabbit skeletal muscle glycogen phosphorylase b (KANDRA et al., 2001). Acceptors were purchased from Sigma; maltotetraose was synthesised from β-cyclodextrin (FARKAS et al., 1997).

Enzymes

α-Amylase (EC 3.2.1.1) from human saliva (Type IXA Sigma) gave a single band on SDS-PAGE and possessed no α- or β-glycosidase activity. The mutant Y151M enzyme was produced as previously described by MISHRA et al. (2002). Methionin has no aromatic ring and OH group therefore Met at position 151 would not provide the stacking interaction and the water-mediated hydrogen-bonding interaction provided by the tyrosine residue of the wild-type enzyme. The Y151M enzyme was purified by a combination of ion exchange chromatography and size exclusion chromatography (data not shown). The SDS-PAGE and western blot analyses of the culture medium showed a very intense band of approximate size 55 kDa corresponding to the recombinant enzyme. As with the expression of wild-type enzyme, hexosamines were not detected in the amino acid analysis indicating that the expressed protein was non-glycosylated. Approximately 5 mg of the protein was finally recovered from a 1 L culture comparable to the amount recovered for HSA. MALDI-TOF MS analysis of the mutant showed a molecular mass of 56.22 kDa corresponding to non-glycosylated enzyme (Fig. 1).

Hydrolysis of maltooligosides

Incubations in 25 mM glycerophosphate buffer (pH 7.0) containing 5 mM calcium acetate and 50 mM NaCl were carried out at 37°C for 2, 5 and 10 min. The reactions were initiated by the addition of enzyme (final concentration of 1.85 nM HSA and 18.8 nM Y151M) to the solution containing 1.0 mM substrate. Samples (20 μL) were taken at the indicated time intervals and the reaction was stopped by the injection of the samples into the chromatographic column. In these studies we have taken care to exclude secondary attack on the products by obtaining the product ratios from early stages of hydrolysis wherein the conversion was always $<10\%$.

Chromatographic analysis

For HPLC a Hewlett-Packard 1090 Series II liquid chromatograph equipped with a diode array detector, automatic sampler and ChemStation was used. The samples were separated on a Spherisorb ODS2 5μm column (250 × 4.0 mm) with acetonitrile:water (13:87) as mobile phase and at a flow rate of 1 mL/min at 40°C. The effluent was monitored for CNP glycosides at 302 nm and the products of the hydrolysis were identified using relevant standards.

Calculation of subsite map

Subsite mapping is simplified for exo-enzymes because there is only one productive binding mode for each substrate. However, endo-enzymes form more productive binding modes resulting in a complex product pattern. The relative rate of formation of each product is called bond-cleavage frequency (BCF), which gives information about subsite-binding energy. By using the BCFs for a series of oligomeric substrates, it is possible to calculate the subsite-binding energy for every subsite on the enzyme-binding region, with the exception of the two subsites adjacent to the catalytic site that are occupied by every productive complex.

A computer program was also used for subsite map calculation (Gyémánt et al., 2002). SUMA (**Su**bsite **M**apping for α-**A**mylases) runs in a Windows^{TM} environment and uses the experimentally determined BCFs for determination of the number of subsites, the position of the catalytic site and for calculation of subsite affinities. The apparent free energy values were optimised by minimisation of the differences between the measured and the primary calculated BCF data. Negative energy values indicate binding (favourable interaction), while positive values indicate repulsion (unfavourable interactions).

General procedure for enzymatic synthesis of 4-nitrophenyl glycoside acceptor products

10 mM maltotetraose and 15 mM arylglycosides were incubated in sodium glycerophosphate buffer (25 mM, pH 6.0) containing 1 mM calcium acetate and 50 mM sodium chloride at 8, 15, 25 and 37 °C for 6, 24, 48 and 72 hours. The reactions were initiated by the addition of Y151M enzyme (20 nM). Samples were taken at different time intervals and the reaction was stopped by 5 minutes boiling. After cooling, the precipitated enzyme was removed by filtration through a Millipore 0.2 μm filter. Samples were analysed by TLC, HPLC and MALDITOF MS. Preparative scale isolation was carried out by semipreparative HPLC. For HPLC, a Merck-Hitachi LaChrom liquid chromatograph equipped with diode array detector, automatic sampler and HPLC System Manager software, was used. Conditions can be found in the *Chromatographic analysis* section.

Kieselgel 60 F_{254} plates were used in $CHCl_3$:MeOH: water = 8:5:1 eluant for TLC. The compounds were detected under UV light and by charring with 50% aqueous sulphuric acid and heating at 120 °C to follow the conversion of maltotetraose.

Mass spectrometry

The MALDI spectra of the compounds were obtained in positive-ion mode using a Bruker Biflex MALDI-TOF mass spectrometer equipped with delayed-ion extraction. Desorption/ionisation of the sample molecules was effected with a 337 nm nitrogen laser. For oligosaccharides, spectra from multiple (at least 100) laser shots were summarized using 19 kV accelerating and 20 kV reflectron voltage. External calibration was applied using the $[M + Na]^+$ peaks of cyclodextrins DP 6-8, m/z: 995, m/z: 1157, m/z: 1319 Da, respectively, where z = 1. The spectrum was obtained with a 2,5-dihydroxy benzoic acid matrix using the dry-droplet method. For proteins, linear spectra were obtained with a sinapinic acid matrix using the standard protocol. External calibration was applied using the $[M+H]^+$ and $[M+2H]^{2+}$ peaks of bovine serum albumin.

^1H and ^{13}C NMR analyses

The anomeric configuration as well as the glycosidic bond type were determined by NMR spectroscopy. ^1H (500.13 MHz) and ^{13}C (125.76 MHz) NMR spectra with D_2O as solvent were recorded with a Bruker DRX-500 spectrometer. Chemical shifts were referenced to external DSS.

Results and discussion

The homologous series of oligomeric substrates and their chromogenic glycosides are of current interest because of their importance in the investigation of the binding site and the action of different depolymerising enzymes. In such studies the well-defined and structurally well-characterised substrates of high purity are preferred. In order to supply a sufficient amount of these enzymologically important substances, highly selective glycosylation reactions are in great demand. Although many glycosylations have already appeared based on recent advances in catalysts for organic synthesis, the perfect control of regio- and stereo-chemistry of the glycosylation process still remains a difficult and challenging problem in glycotechnology. Enzymes have several remarkable catalytic properties compared with other types of catalysts in terms of their selectivity, high catalytic activity, lack of undesirable side reactions and operation under mild conditions. Recently, a chemoenzymatic procedure was developed in our laboratory for the synthesis of 2-chloro-4-nitrophenyl β-maltooligosides in the range of DP 3-11 (Kandra et al., 2001), and these compounds were used as model substrates for the analysis of action pattern of amylases (Kandra et al., 2002a,b).

Compared with other substrate series reported so far, for example, maltooligosaccharides (Haegele et al., 1981) or α-NP-maltooligosaccharides (MacGregor et al., 1992), the CNP-maltooligosaccharides, which are β-glycosides, are unique since the β-linkage is stable and is not hydrolysed by α-amylases. Therefore, the reducing-end products of hydrolysis are always β-glycosides and lead to clear identification, using UV detection, of the site of cleavage.

Action pattern and cleavage frequencies of HSA and Y151M on CNP-maltooligosides

The series of CNP-maltooligosaccharides DP 3-10 was used as substrates for HSA and Y151M. The products were analysed using HPLC to determine unambiguously the exact glycosidic linkage being cleaved, as well as the cleavage frequency, an indicator of the binding mode of the corresponding substrate. Table 1 summarizes the product ratios for HSA and Y151M.

The product distribution for the Y151M mutant, on the same oligosaccharide series, was very interesting and markedly different from that of HSA. The moiety CNP-G1 is the major product of hydrolysis when CNP-G3 and CNP-G4 were used as substrates. Thus, hydrolysis of CNP-G3, CNP-G4 and CNP-G5 resulted

Table 1. Yields of products from the hydrolysis of CNP-maltooligosaccharides by HSA and Y151M (KANDRA et al., 2003)[a].

Substrates	Products (mol %)						
	CNP-G1	CNP-G2	CNP-G3	CNP-G4	CNP-G5	CNP-G6	CNP-G7
CNP-G3	–	–					
	77	**23**					
CNP-G4	10	85	5				
	76	**17**	**7**				
CNP-G5	2	86	12	–			
	34	**54**	**4**	**8**			
CNP-G6	–	51	44	5			
	10	**63**	**26**	**1**			
CNP-G7	–	18	50	30	2		
	8	**25**	**33**	**34**	–		
CNP-G8	–	16	27	41	16		
	10	**18**	**17**	**38**	**17**		
CNP-G9	–	17	19	26	30	8	
	8	**30**	**14**	**18**	**24**	**6**	
CNP-G10	–	17	29	16	11	19	8
	7	**16**	**25**	**11**	**11**	**15**	**15**

[a] Bold numbers indicate the bond cleavage frequencies of Y151M enzyme.

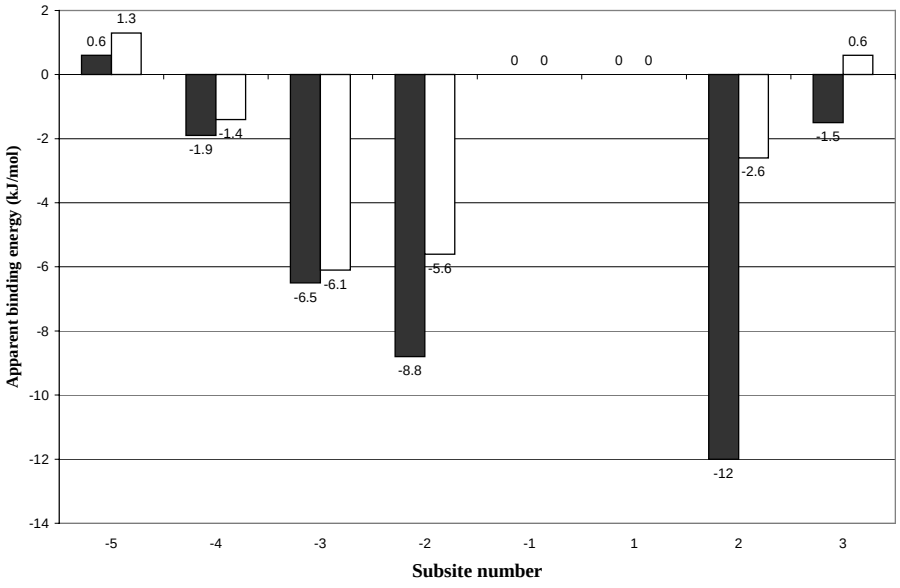


Fig. 2. Subsite maps for human salivary α-amylase (HSA; solid bars) and its Y151M mutant (open bars). The location of the bond cleavage is between subsites -1 and $+1$. The reducing end of maltooligomers would be situated at the right hand of the subsite map. Negative energy values indicate binding between the enzyme and aligned glucopyranosyl residues, while positive values indicate repulsion (KANDRA et al., 2003).

in 77%, 76% and 34% CNP-G1 compared to 0%, 10% and 2% CNP-G1 when HSA was used as a catalyst. There is a remakable reduction in the amount of CNP-G2 produced in the hydrolysis of CNP-G4 and CNP-G5 by Y151M compared to HSA (85% and 86% CNP-G2, respectively, for HSA vs. 17% and 54%, respectively, for Y151M). This suggests a favourable interaction between the glucose moiety and subsite (+1), but a less favourable one between a glucose residue and the subsite (+2). The moiety CNP-G1 was significantly released in the hydrolysis of longer oligomers (DP 6-10) as well, whereas this monomer glycoside was not recognisable as a product in the hydrolysis of the corresponding substrates by HSA. In the mutant, the point of cleavage moved closer to the reducing end by one subsite resulting in a clear shift in the action pattern. This tendency is evident for all of the substrates DP 3-10. These results can be explained by the presence of methionine at subsite (+2), which is not advantageous for the binding of the polar glucose residues.

Subsite mapping of HSA and Y151M

For graphical evaluation of a subsite map, the apparent binding energies of subsites are plotted in a block diagram. Figure 2 shows the apparent binding energies of subsites for HSA and Y151M. Surprisingly, the calculated binding energy at subsite (+2) (-12.0 kJ/mol) indicates a remarkably good interaction with the bound monomer unit compared with the other subsite energies. This finding is in a good agreement with the three-dimensional structural model of HSA described by RAMASUBBU et al. (2003). Although a decreased binding energy was envisaged for subsite (+2) in Y151M mutant, all of the subsites exhibited a lower affinity for

Table 2. Consequences of mutation.

Parameters	Enzymes	
	Wild HSA	Mutant
Amino acid at (+2)	Tyr151	Met151
Binding energy at (+2) (kJ/mol)	−12	−2.6
Preferred minimal leaving group	Maltose	Glucose
Subsite model	4+3	4+2
k_{cat}/K_M (1/s.mM)	336	29
Hydrolase/transferase activity	100:1	2:1

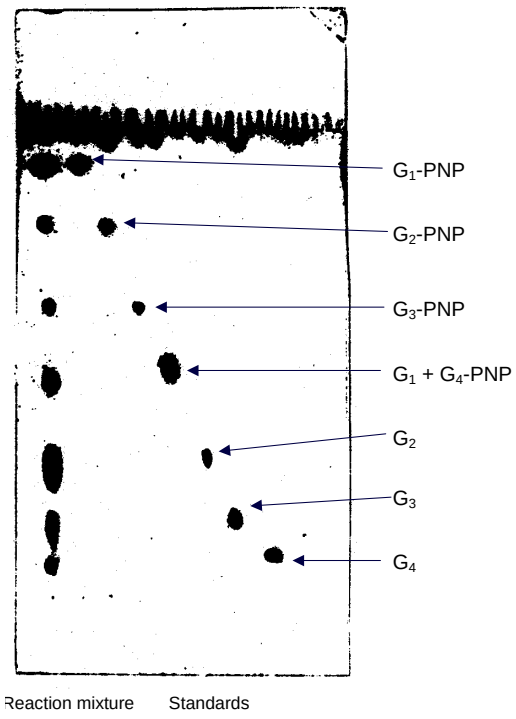


Fig. 3. TLC of reaction mixture of maltotetraose donor and PNP-glucoside acceptor catalysed by Y151M. Eluant: $CHCl_3$:MeOH: water = 8:5:1.

a glucose residue compared to the corresponding subsites of HSA. Such a reduction fits well with cooperative binding of the oligosaccharide ligand. The most remarkable decrease in the calculated binding energies can be found at subsite (+2): −2.6 kJ/mol compared to −12.0 kJ/mol when HSA was used. These findings confirm clearly the role of Tyr151 at subsite (+2) and provide evidence that stacking interactions at the reducing end are important in substrate binding and product distribution in the hydrolysis of oligosaccharides catalysed by HSA (Ramassubu et al., 2005).

Effect of mutation on HSA

Consequences of mutation are summarised in Table 2. The product distributions revealed that in the mutant maltose changed to glucose as the preferred minimal leaving group due to the presence of Met at subsite

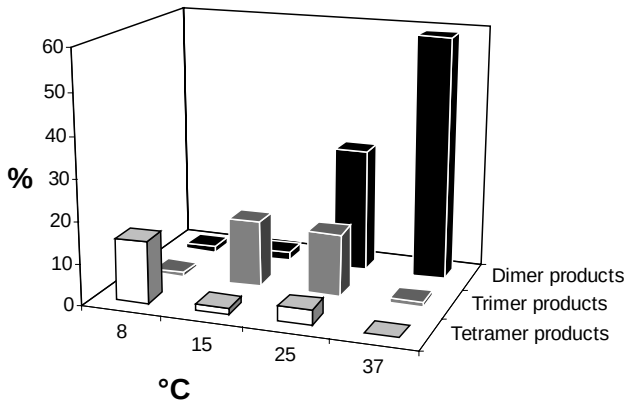


Fig. 4. Effect of temperature on product distribution in the presence of maltotetraose donor, PNP-1-S-glucoside acceptor and Y151M catalyst.

(+2), which is apparently not advantageous for polar glucose residue binding. Thus the binding region of the mutant is shorter with one aglycone-binding site. The binding energy of subsite (+2) decreased significantly in the mutant. In addition, the mutant showed a significant reduction in its hydrolytic efficiency and a remarkably good increase in its transglycosidase activity. Transglycosylation was carried out using maltotetraose as donor and PNP-α-glycoside as acceptor. The reaction products were separated by TLC (Fig. 3). It was envisaged that the structural change at the aglycone-binding site could improve the synthetic activity of HSA, and p-nitrophenyl-glycosides would be better acceptors for Y151M than for HSA. Using PNP as an aglycone is very advantageous since it makes detection and differentiation of reaction products more sensitive and unambiguous.

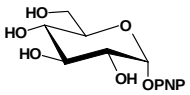
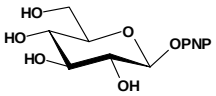
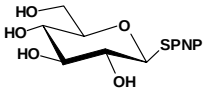
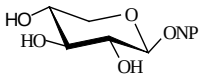
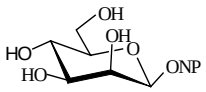
Taking advantage of the transferase activity of the mutant, an enzymatic procedure was developed for the synthesis of 4-nitrophenyl oligosides with a DP from 2 to 4. The acceptors used and the product distributions of the transglycosylation are shown in Table 3. The PNP α-Glc, PNP β-Glc and PNP 1-S-β-Glc acceptor products were formed with similar good yield, 48.8%, 40.0% and 41.0% conversion, respectively. PNP 1-S-β-Glc was selected for further detailed investigations (Remenyik et al., 2003).

Effect of temperature and time on control of transglycosylation

The temperature range covered was between 8 and 37 °C. The transfer products were monitored by HPLC after 6 hours incubation time. The distribution of products is shown in Figure 4.

Incubation at 8 °C resulted in the dominant formation of PNP 1-S-β-Glc_4 in a yield up to 15%, while at 15 °C PNP 1-S-β-Glc_3 was the major acceptor product with a similar yield. Enzymatic transglycosylation at 25 and 37 °C resulted in the dimer glycoside as the main product (30% and 59%, respectively). Dimer glycoside

Table 3. Products of transglycosylation[a] catalysed by Y151M.

Acceptors	Products (Area %)				Conversion (%)
	Dimer	Trimer	Tetramer	Pentamer	
PNP-α-Glc 	30	15	3.8	–	48.8
PNP-β-Glc 	29	10	1	–	40
PNP-1-S-β-Glc 	30	10	1	–	41
PNP-β-Xyl 	15	7	5	0.8	27.8
PNP-β-Man 	3	2	–	–	5

[a] Maltotetraose was used as a donor. General reaction conditions can be found in Materials and methods. Reaction time: 6 h; temperature: 37 °C.

Table 4. Time course of transglycosylation[a] at 8 °C.

Time (h)	Yields of products (Area %)		
	Tetramer	Trimer	Dimer
6	16.5	1	1.5
24	3	17	16
48	2	15	18
72	2.2	14	19

[a] Conditions are described in Materials and methods.

might be a secondary hydrolysis product from higher oligosaccharide glycosides.

Since tetramer glycoside was formed at 8 °C, this low temperature was selected to study the time course of reaction (Table 4). During longer incubation times, trimer and dimer glycosides were obtained due to the hydrolysis of tetramer. The rate of hydrolysis of trimer glycoside is low, while dimer is resistant to hydrolysis by Y151M; therefore dimer and trimer can accumulate.

Structural analysis of the PNP 1-thio-D-maltoside

Since the dimer can be obtained in very good yield because it is not hydrolysed by the Y151M enzyme, the products were separated by preparative HPLC on a reversed phase column using acetonitrile:water (13:87) as eluant. Structural parameters of the purified dimer were established by MALDI-TOF MS analysis (Fig. 5) and ^{13}C and ^{1}H NMR spectroscopy (Table 5).

The identity of the product was verified to be the dimer PNP 1-S-β-Glc$_2$ based on the good agreement between the calculated (502.10 Da) and measured (502.14 Da) molecular masses from the MALDI-TOF MS spectrum. Since PNP aglycone has an absorption maximum at 302 nm, it can therefore absorb the energy of laser light (337 nm) that causes appearance of fragment peaks at m/z=486–490 in the MALDI spectrum.

Table 5. ^{1}H-NMR and ^{13}C-NMR data of the PNP-1-thio-β-D-maltoside.

C	δ^{1}H (ppm)	δ^{13}C (ppm)	J (Hz)
1	5.02	**85.14**[a]	
2	3.53	71.15	$J_{1,2}$ = 10.3
3	3.87	77.25	
4	3.76	*78.18*[a]	
5			
6		60.38	
1'	5.36	**99.22**	
2'	3.6	71.25	$J_{1',2'}$ = 3.5
3'	3.71	72.44	
4'	3.45	*68.00*	
5'			
6'		60.05	

[a] Values in bold and italic numbers are explained in the text.

The ^{1}H-NMR spectrum revealed the presence of two doublets with 3.5 and 10.3 Hz J1,2 coupling constants. The 10.3 Hz value can be assigned to the β-glycosidic linkage; the other one belongs to the newly formed α-interglycosidic bond. These data verify that the Y151M mutated enzyme retained its α-stereoselectivity and did not alter the anomeric con-

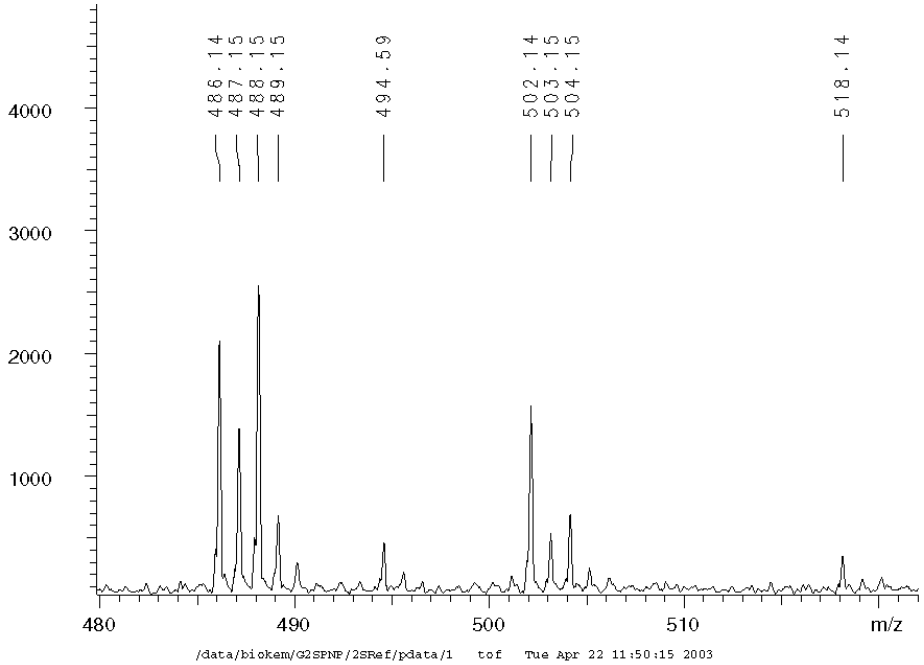


Fig. 5. MALDI-TOF spectrum of purified 4-nitrophenyl 1-thio-β-D-glucopyranosylidene-α-D-(1-4)-glucopyranoside (Remenyik et al., 2003).

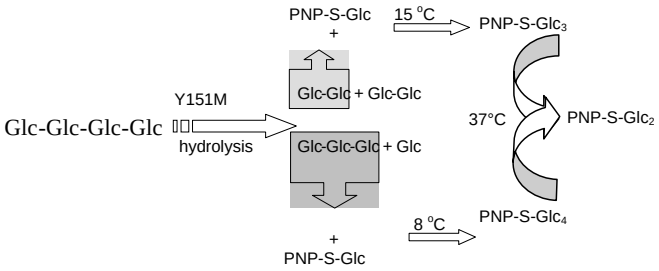


Fig. 6. Schematic representation of the suggested reaction of maltotetraose and PNP 1-S-β-Glc catalysed by Y151M.

figuration of the acceptor molecule. These conclusions were supported by the chemical shift values of the C-1 and C-1' in the ^{13}C-NMR spectrum at 85.14 and 99.22 ppm, respectively.

We found that the C-4 has the highest chemical shift value (78.18 ppm) among the skeleton carbons with the exception of the two anomeric carbons. This confirms the presence of an α-1,4-glycosidic bond and suggests that Y151M used for glycosylation retained not only its stereospecificity but also its regioselectivity. In conclusion, the disaccharide glycoside can be described as an α-D-Glcp(1→4)-β-D-Glcp-(1→SPNP).

Conclusion

The transglycosylation procedure is summarised in Figure 6. The enzyme is capable of transferring maltose and maltotriose onto PNP 1-S-β-Glc acceptor resulting in PNP 1-S-β-Glc$_3$ and PNP 1-S-β-Glc$_4$ products, respectively. Enzymatic hydrolysis of PNP 1-S-β-Glc$_4$ leads to the formation of PNP 1-S-β-Glc$_2$. Dimer and trimer products accumulate because of their very slow hydrolysis.

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